Dysregulated Expression of COOH-Terminally Truncated Stat5 and Loss of IL2-Inducible Stat5-Dependent Gene Expression in Sezary Syndrome

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ABSTRACT

Sezary Syndrome (SzS) is a leukemic variant of cutaneous T-cell lymphoma characterized by the accumulation of clonal neoplastic CD4+ T cells. The signal transducers and activators of transcription (STAT) family members, Stat5a and Stat5b, play an important role in regulating T-cell activation. Recent studies have shown that inappropriate activation of STATs occurs frequently in a wide variety of human cancers. Here we examine the functional status of Stat5 proteins in SzS as compared with healthy donors. Western blotting demonstrates that in cytoplasmic extracts of unstimulated T cells from healthy controls two isoforms of Stat5, full-length and a COOH-terminal truncated isoform, termed Stat5t, are present. However, bandshift assays demonstrate that only Stat5t, translocates to the nucleus and binds DNA on IL-2 stimulation. In contrast, preactivated T cells express only full-length Stat5, which is functionally activated on IL-2 stimulation. Analysis of Stat5 proteins from five of five SzS patients revealed predominant aberrant expression of Stat5t in preactivated peripheral blood mononuclear cell. Furthermore, patients showed preferential IL-2-induced DNA binding of Stat5t. Consistent with the inappropriate activation of Stat5t in SzS patients, real-time PCR revealed that IL-2-induced mRNA expression of the Stat5 target genes, Bcl-2, PIM-1, and CISH were markedly reduced. These data indicate that functional Stat5 isoform expression is regulated by T-cell activation status and that dysregulated expression of Stat5t, in malignant T cells in SzS can suppress Stat5-dependent gene expression. Thus, aberrant expression of Stat5t may be one mechanism that contributes to the cellular transformation of T cells in this disease.

INTRODUCTION

Primary cutaneous T-cell lymphomas are the second most common form of extranodal lymphoma. Mycosis fungoides and its erythrodermic variant Sezary Syndrome (SzS), are the most frequent cutaneous T-cell lymphomas involving the skin. Whereas mycosis fungoides presents with a skin-restricted infiltration of clonal T cells and an indolent course, SzS is an aggressive form of cutaneous T-cell lymphomas associated with the disseminuation of malignant T cells to the lymph nodes and peripheral blood. A typical cytogenetic feature of many SzS patients is the presence of multiple chromosomal abnormalities, involving specific chromosomes, including 1, 10, 17, 18, and 19 (1–3). Although the identities of the genes that are affected by any of the chromosomal aberrations have not been elucidated, studies of candidate genes, particularly those involved in cytokine signaling pathways, cell proliferation, and survival have enhanced our understanding of the molecular basis of SzS.

One important family of genes located on the long arm of chromosome 17 encodes the signal transducers and activators of transcription (STAT) proteins, Stat5a, Stat5b and Stat3, all of which are involved in signal transduction by the IL-2-family of cytokines and, thus, play a central role in regulating the immune response. Whereas chromosomal aberrations of STAT genes have not been reported in SzS, dysfunction of STAT proteins and/or their activating kinases are likely candidates that may contribute to the cellular transformation and the altered cytokine responsiveness of malignant T cells in this disease (4–6).

Stat5 is encoded by two homologous genes, Stat5a and Stat5b, which share 96% identity at the protein level and diverge at their COOH termini (7–10). Their unique and overlapping functions, which are essential to a variety of signaling pathways, have been demonstrated by targeted gene deletion studies (11–20). Additionally, several studies involving cell transformation, either by expression of oncogenes or virally induced tumors, have revealed that this process often involves the constitutive activation of STAT proteins, particularly Stat3 and Stat5 (21–23). It is thought that their constitutive activation is necessary to achieve cytokine independence during tumor development.

Constitutive activation of Stat3 has been demonstrated previously in a tumor cell line obtained from peripheral blood mononuclear cell (PBMC) of a patient with SzS, and was shown to functionally contribute to the elevated levels of high-affinity IL-2R expression (IL-2Rαβγ) seen in these tumor cells by activating IL-2Rα chain gene expression (24). In light of these findings, it was compelling to evaluate the functional status of Stat5 proteins in SzS, as both Stat5a and Stat3 are involved in the IL-2-inducible activation of the IL-2Rα gene (12, 25–29). Although a previous study had identified weak basal phosphorylation of Stat5 in fresh PBMCs from SzS patients, it was shown to be the result of elevated cycling levels of cytokines rather than constitutive phosphorylation (30). To date, activation of aberrant Stat5 proteins in SzS patients has not been reported.

In this study, we have compared Stat5 protein expression and function in PBMCs from healthy individuals and SzS patients. We report that in fresh PBMCs and T cells isolated from healthy individuals, only the truncated isoform of Stat5, Stat5t, is expressed in the nucleus, whereas cytoplasmic extracts from these cells contain both full-length and Stat5t isoforms. After mitogenic activation of PBMCs and T cells from healthy controls, expression of Stat5t is down-regulated, and replaced by expression of full-length Stat5 protein in both nuclear and cytoplasmic subcellular fractions. In contrast, pre-activated PBMCs derived from all five SzS patients revealed substantially elevated aberrant expression of Stat5t, IL-2 treatment of PBMC from SzS patients, but not controls, resulted in the activation and predominant DNA binding of Stat5t, rather than full-length Stat5. Finally we demonstrate that consistent with the aberrant expression of Stat5t, IL-2-induced Stat5-dependent gene expression is abrogated in SzS patients compared with that observed in healthy individuals.

MATERIALS AND METHODS

Preparation and Treatment of PBMCs from Patients and Healthy Donors. The five patients (P1–P5) included in this study fulfilled the criteria for a diagnosis of SzS (31). Clinical and immunophenotypic features included erythroderma, pruritus, and lymphadenopathy and the presence of Sezary cells in peripheral blood, at a relative amount of 38% (P1), 31% (P2), 21% (P3), 44% (P4), and 40% (P5) of the total lymphocyte count, and all had compatible skin histology. Furthermore, the presence of a T-cell clone in peripheral blood using T-cell receptor gene analysis (32) was also demonstrated in all five of the
patients. Eight healthy adults served as controls for this study. PBMCs were prepared according to the manufacturer’s recommendations by centrifugation of Ficoll/Hypaque gradients (Sigma Chemicals). Fresh PBMCs were cultured in RPMI 1640 (BioWhittaker) containing 10% fetal bovine serum (BioWhittaker), 2 mM glutamine, and 100 units/ml penicillin and streptomycin, and then either left untreated (control) or treated with 100 units/ml recombinant human IL-2 (Roche Biochemicals) for 30 min. Preactivated PBMCs were prepared by culturing PBMCs in 2.5 μg/ml phytohemagglutinin (Amersham Pharmacia-LKB) for 3 days and then rested overnight in fresh medium for 16–20 h. Preactivated PBMCs were then either treated with 100 units/ml IL-2 for 30 min, or left untreated. T cells were purified from normal healthy donors using nylon wool by standard methods. The purity of isolated T cells was assessed by flow cytometry analysis (fluorescence-activated cell sorter), using CD3-PE (Dako), CD19-FITC (Dako), CD45-Cy5 (Coulter) antibodies, and mouse IgG1-FITC + IgG1 RPE + IgG1 RPE-Cy5 (Dako) as a negative control. The percentage of T cells recovered from the purification was 78%.

Cell Lines. YT, a human NK cell line (35), kindly provided by J. Yodoi (Kyoto University, Kyoto, Japan) was grown in complete RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum (BioWhittaker).

Whole Cell Extracts, Nuclear Extracts, and Electrophoretic Mobility Shift Assays (EMSAs). Whole cell extracts were prepared essentially as described (34). Briefly, cell pellets were solubilized directly by the addition of 10 volumes of boiling SDS sample buffer [62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue], and then boiled for 10 min. Nuclear extracts were prepared from fresh PBMCs or preactivated PBMCs as described (10). All of the buffers used in the nuclear extract preparations and EMSAs contained protease inhibitor mixture-I, (Calbiochem), 1 mM sodium orthovanadate, and 10 mM sodium fluoride. EMSAs were performed using 10 μg of nuclear extracts and 20,000 cpm of 32P-labeled DNA probe. The probe used in EMSAs was the −3780 to −3737 fragment of IL-2 response element of the IL-2 receptor α chain promoter (PRRRIII), containing tandemly linked IFN-γ-activated sequences motifs (GAS) (25): 5′-GAGCGATTCTT-TCAGAGAGTACAAACATTCTGTGATAAGA-3′. HindIII and BamHI restriction endonuclease sites were added to the 5′- and 3′-ends of the oligonucleotide, respectively, to facilitate labeling of the probe by end-filling reaction using the Klenow fragment of DNA polymerase I (New England Biolabs). DNA-supershifts were performed by preincubating 10 μg nuclear extracts with 0.5 μg of anti-Stat5α or anti-Stat5β-specific monoclonal antibodies (Zymed) or 1 μg of pan-Stat5 antibody (Santa Cruz Biotechnology) for 30 min before the addition of probe, followed by an additional incubation of 20 min on ice after addition of probe.

SDS-Gel Electrophoresis and Western Blot Analysis. Ten μg of nuclear extracts were separated on 8% or 10% Tris-Glycine gels (Novex-Invitrogen Life Technologies, Inc. Ltd.) and transferred to polyvinylidene difluoride paper (Immobilon). Blots were incubated with anti-Stat5α or anti-Stat5β-specific antibodies (Zymed), pan-Stat5 antibody (PharMingen-Transduction Laboratories), and developed using SuperSignal chemiluminescent detection reagents (Pierce Chemicals).

RNA Preparation and First-Strand cDNA Synthesis. Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen). A total of 2 μg of total RNA was reverse transcribed using oligo(dT)12-18 primer and Omniscript (Qiagen) reverse transcriptase according to the manufacturer’s instructions.

Quantitative Real Time Reverse Transcription-PCR. Real-time PCR was performed in 96-well plates on the ABI Prism 7000 Sequence Detection System (ABI) data collection, and analyses were performed using the manufacturer’s software. Two-step reverse transcription-PCR was performed using dilutions of first-strand cDNA with a final concentration of 1× Assays-On-Demand and 1× TaqMan Universal PCR Master Mix (ABI P/N 4304437). The final reaction volume was 25 μl. Each sample was analyzed in triplicate. All of the experiments were repeated twice. A nontemplate control (RT-negative water) was included on every plate. The thermal cycler conditions were 2 min hold at 50°C (UNG activation), 10 min hold at 95°C, followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension).

In the first instance a standard curve and validation experiment was performed for each primer/probe set. A series of 7 serial dilutions of YT cDNA were used as a template for each primer/probe set. Standard curves were generated by plotting the threshold cycle (Ct) value numbers against the log of the amount of input cDNA. Ct is the PCR cycle at which an increase in reporter fluorescence above the baseline level is first detected. Each target gene was plotted with the endogenous control (cyclophilin). The absolute value of the slope of log input amount versus Δ Ct was <0.1 with all four of the target genes (Bcl-2, PIM1, CISH, and CD25). This indicates that the relative efficiencies of all of the primer/probe sets are approximately equal and, therefore, the comparative Ct method of data analysis was used to analyze the data. Comparative Ct uses arithmetic formulae to calculate relative quantitation of the target gene expression. The amount of target gene expressed is normalized to an endogenous reference and is relative to a calibrator. The endogenous reference used in all of the experiments reported here was cyclophilin. A control phytohemagglutinin-stimulated PBMC CDNA was used as the calibrator in all of the experiments. The target Ct and endogenous control Ct was calculated for each sample (analyzed in triplicate). The Ct of the endogenous control is then subtracted from the Ct of the target gene. This value is known as Δ Ct. The Δ Ct of each sample is then subtracted from the Δ Ct of the calibrator, and this value is known as ΔΔ Ct. The amount of target gene expression normalized to the endogenous control and relative to the calibrator is calculated using the formula 2−ΔΔ Ct. The average and SD of 2−ΔΔ Ct was calculated for the triplicate measurements, and the relative amount of target gene expression for each sample was plotted in bar charts using Microsoft Excel software.

Primers and Probes for Quantitative Real-Time Reverse Transcription-PCR. PCR primers and fluorogenic probes for all of the target genes and endogenous controls were purchased as Assays-On-Demand (ABI, Foster City, CA). The assays are supplied as a 20× mix of PCR primers and TaqMan minor groove binder 6-FAM dye labeled probes with a nonfluorescent quencher at the 3′ end of the probe. The assays are optimized for use on any ABI PRISM Sequence Detection System using the default machine settings. The assay numbers for the endogenous control (cyclophilin) and target genes were as follows: Hs99999904 ml (Cyclophilin); Hs001153350-m1 (Bcl-2); Hs00171473 ml (PIM-1); Hs00367082 ml (CISH); and Hs00166229 ml (CD25).

RESULTS

Fresh PBMCs from Healthy Donors Only Express COOH-Terminally Truncated Forms of Stat5 in the Nucleus. To investigate the activation of Stat5 proteins in response to IL-2 stimulation, we studied fresh and preactivated PBMCs and purified T cells. Western blot analysis of nuclear extracts derived from fresh and preactivated PBMCs from healthy donors that were untreated or treated with IL-2 revealed that the majority of the IL-2-induced Stat5 protein in fresh PBMCs migrated at approximately M r 70,000–80,000 (Stat5α), whereas preactivated PBMCs primarily expressed “full-length” Stat5 proteins of approximately M r 96,000–92,000 (Fig. 1A, Lanes 1–4). As expected, IL-2 rapidly induced nuclear translocation of activated proteins, resulting in increased levels of Stat5 protein, seen in the presence of IL-2 compared with the untreated samples (Fig. 1A, Lanes 2, 4, 6, and 8). Nuclear Stat5 proteins were observed in the unactivated samples (see Fig. 1A, Lanes 1, 3, 5, and 7), suggesting that Stat5 proteins translocate to the nucleus in the absence of cytokine stimulation. This observation is consistent with the presence of specific protein motifs that regulate cytokine-dependent and independent nuclear import/export of Stat5 proteins (35–37).

Using Stat5a and Stat5b-specific antibodies with reactivities to the COOH terminus of each protein, we observed that the Stat5, isoform, which is expressed in fresh PBMCs, could not be detected, suggesting that this isoform of Stat5 is truncated at the COOH terminus (Fig. 1A, Lanes 1 and 2, Stat5a and Stat5b panels). Full-length Stat5 proteins from preactivated PBMC extracts, were detected by all three of the antibodies (Fig. 1A, Lanes 4 and 8, Stat5a and Stat5b panels). As similar observations of COOH-terminally truncated Stat5 proteins have been reported

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Predominant Expression of the Truncated Form of Stat5 in PBMCs from SzS Patients. Western blot analysis of fresh PBMC-derived nuclear extracts demonstrated that SzS patients examined predominately expressed Stat5t at comparable levels to those observed in the normal control samples (Fig. 2, A–E, Lanes 1 and 2 versus Lanes 5 and 6). However, in contrast to the control samples Western blot analysis of nuclear extracts from preactivated PBMCs revealed that all five of the patients examined substantially elevated or predominant expression of Stat5t rather than full-length Stat5 as in control samples (Fig. 2, A–E, Lanes 3 and 4 versus Lanes 7 and 8). This defect was most pronounced in patient 2 who revealed almost exclusively Stat5t expression (Fig. 2B), whereas patients 1 and 3–5, revealed numerous amounts of full-length Stat5 and significantly greater levels of Stat5t, compared with control samples (Fig. 2, A and C–E). Indeed, of the four different healthy individuals only one sample revealed low levels of Stat5t expression in preactivated PBMC nuclear extracts (Fig. 2C, Lanes 7 and 8). Thus, T cells from SzS patients displayed a marked inability to express full-length Stat5 proteins in the nucleus even after potent activation. These data suggest that the regulation of expression of transcription-competent, full-length Stat5 isoforms in response to potent T-cell activation signals is grossly dysregulated in malignant T-cells from SzS patients. Despite these changes in the nuclear expression of Stat5 isoforms, none of the patients showed constitutive Stat5 phosphorylation that was significantly different from control samples (data not shown).

Changes in Stat5 Expression Result in an Altered Pattern of DNA Binding in SzS Patients. We investigated the ability of Stat5 from SzS patients to bind to naturally occurring GAS motifs from target gene promoters. We used the tandemly linked GAS motifs from the S'-promoter region of the human IL-2 receptor alpha promoter, which is regulated by the binding of tetrameric STAT5 protein complexes (41). Similar results were obtained with the high-affinity GAS motif derived from the β-casein promoter (data not shown).

Using the IL-2 receptor alpha probe, control fresh PBMC extracts exhibited binding of two IL-2-inducible complexes, both of which contain Stat5 proteins, as indicated by the antibody-mediated supershift of both protein-DNA complexes by an NH2-terminal, pan-Stat5 antibody.

Previously in immature myeloid and natural killer cell populations (38–40), we confirmed that our observations reflected the situation in the T-cell population, and not contaminating myeloid or natural killer cells by analyzing the Stat5 isoform expression in purified T cells (Fig. 1A, Lanes 5–8). Nuclear extracts prepared from the T-cell subset and analyzed by Western blot analysis as above revealed an identical pattern of Stat5 isoform expression in fresh versus preactivated T cells, as seen with fresh PBMCs (Fig. 1A, compare Lanes 1–4 with Lanes 5–8). Thus, in healthy individuals, Stat5 and not full-length Stat5 proteins are activated in response to IL-2 stimulation in fresh PBMCs/T cells, whereas the full-length Stat5 is predominantly expressed in the nucleus only once the cells are maximally activated.

We next examined whether full-length Stat5 proteins were expressed in the cytoplasm of PBMCs. Cytoplasmic and nuclear extracts were prepared from uninduced or IL-2-treated fresh and preactivated PBMCs and separated by SDS-PAGE, followed by Western blot analysis using a pan-Stat5 antibody (Fig. 1B). Unlike nuclear extracts, cytoplasmic extracts from fresh PBMCs contained both the full-length Stat5 and Stat5t proteins, suggesting that the full-length protein is cleaved in the cytoplasm, and only the truncated form translocates to the nucleus (Fig. 1B, Lanes 1–4). To exclude the possibility that Stat5t was generated by experimental artifacts during sample preparations, we also prepared whole cell extracts by directly lysing the cell pellets in boiling SDS-sample buffer. Both forms of Stat5 proteins were also observed in these extracts, confirming the physiological production of Stat5, in fresh PBMCs (Fig. 1B, Lanes 5 and 6). In contrast, Western blot analysis of both cytoplasmic and nuclear extracts prepared from preactivated PBMCs using the pan-Stat5 antibody detected predominantly full-length Stat5 proteins (Fig. 1B, lanes 7–10). These data suggest that the activation status of PBMCs regulates the differential expression of Stat5 isoforms in the nucleus.

Fig. 1. Differential usage of Stat5 isoforms in fresh and preactivated PBMCs from healthy donors. A, nuclear extracts from untreated or IL-2-treated fresh (Lanes 1, 2, 5, and 6) and preactivated (Lanes 3, 4, 7, and 8) PBMCs (Lanes 1–4) or purified T cells (lanes 5–8) from a normal healthy donor analyzed by Western blot analysis for Stat5 protein expression using a pan-Stat5 antibody (top panel) or Stat5a (middle panel) and Stat5b (bottom panel) specific antibodies. B, cytoplasmic (CE), nuclear (NE), or whole cell extracts from fresh (lanes 1–6) and preactivated PBMC (7–12) from normal healthy donors and analyzed by Western blot analysis using a pan-Stat5 antibody.

Fig. 2. Nuclear Stat5 isoform expression in SzS patients. A–E, Western blot analysis was performed on fresh and preactivated PBMCs, untreated or IL-2-treated nuclear extracts from 5 SzS patients (as indicated on each panel, Lanes 1–4), and four different control healthy donors (Lanes 5–8), using a pan-Stat5 antibody in all cases. In all panels, A–E, Stat5t refers to the COOH-terminally truncated isoforms of Stat5.
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Fig. 3. IL-2-inducible activation and binding of truncated Stat5 isoforms in fresh PBMCs and full-length Stat5 isoforms in activated PBMCs. A, fresh (Lanes 1–5) and preactivated PBMCs (lanes, 6–10), and nuclear extracts from a control healthy donor were used in EMSAs using the IL2-response element from the IL-2Rα chain gene as the DNA probe. The IL-2-inducible complexes and the reactivity of these complexes to the pan-Stat5 (fresh and preactivated PBMCs, Lanes 3 and 8) or Stat5a and Stat5b-specific antibodies (preactivated PBMCs, Lanes 4, 5, 9, and 10) are indicated. B, similar analysis as in A was performed on fresh PBMC nuclear extracts treated or untreated with IL-2, from a control donor (Lanes 1–5) or representative patient samples (Lanes 6–9). Supershift analysis using a pan-Stat5 antibody reveals the presence of Stat5 in the IL-2-induced complexes (Lanes 3, 6, and 9). EMSAs were performed on IL-2-induced preactivated PBMC nuclear extracts from a control healthy donor (Lanes 1–5) and the same patient samples as in B (Lanes 6–15). Reactivities to pan-Stat5 (Lanes 3, 8, and 13), Stat5a (Lanes 4, 9, and 14), and Stat5b (Lanes 5, 10, and 15)-specific antibodies were assessed for all samples to determine the relative activation and binding of truncated Stat5 versus full-length Stat5.

(Fig. 3A, Lanes 1–3). The inducible two complexes may correspond to binding of different truncations of Stat5 proteins or may represent tetrameric and dimeric Stat5-DNA complexes as described previously (41). However, neither complex revealed any reactivity to COOH-terminal Stat5a or Stat5b-specific antibodies (Fig. 3A, Lanes 4 and 5, respectively). Thus, in fresh PBMCs from healthy individuals, IL-2 induces the activation of Stat5, to bind to the promoters of target genes.

Preactivated PBMC nuclear extracts from controls primarily show strong IL-2-inducible binding of the slow mobility complex, and greatly diminished, if any, binding of the faster mobility complex (Fig. 3A, Lanes 6 and 7). However, in contrast with fresh PBMCs and consistent with the presence of full-length Stat5 proteins in preactivated PBMCs, supershift analyses using the Stat5a and Stat5b-specific antibodies revealed reactivity of the IL-2-induced complexes to both these antibodies in addition to the pan-Stat5 antibody (Fig. 3A, Lanes 8–10). Thus, activation of T cells results in the synthesis and IL-2-induced functional activation of the full-length Stat5 isoforms.

Similar experiments were performed using fresh and preactivated PBMCs from the five SzS patients. Data are shown for two representative patient samples (Fig. 3B). As noted above with control samples, two new IL-2-inducible protein-DNA complexes were observed in fresh PBMC nuclear extracts from SzS patients, albeit at various levels (Fig. 3B, Lanes 2, 5, and 8). DNA supershifts generated by reactivity to the pan-Stat5 antibody, confirmed that in all of the cases the inducible complexes contained IL-2-induced Stat5 protein (Fig. 3B, Lanes 3, 6, and 9). As shown for healthy control fresh PBMCs (Fig. 3A, Lanes 4 and 5), none of the IL-2-induced Stat5-DNA complexes in fresh PBMC extracts from patients 1–5 reacted with the Stat5a- or Stat5b-specific antibodies, confirming the absence of full-length Stat5 proteins in the IL-2-induced complexes (data not shown).

We next investigated the status of Stat5 binding in the preactivated PBMCs of patients as compared with controls (Fig. 3C). As with the fresh PBMC analysis, data from the same two representative patient samples are shown. In control and patient samples, an IL-2-induced complex was observed, confirming the inducible rather than constitutive activation of Stat5 proteins in all of the cases (Fig. 3C, Lanes 1, 2, 6, 7, 11, and 12). The IL-2-induced complex obtained with the control extract migrated with a slower mobility compared with that seen with patient samples suggesting that the proteins in the two complexes are of potentially different sizes (Fig. 3C, compare Lanes 2, 7, and 12). A pan-Stat5 antibody supershifted the IL-2-induced DNA-protein complexes from control and patient samples, confirming the presence of Stat5 protein in these complexes (Fig. 3C, Lanes 3, 8, and 13). Consistent with the observation of the difference in mobilities of the IL-2-induced complex between the patient and control samples, the mobilities of the supershifted complexes were also correspondingly different. Importantly, whereas the Stat5a and Stat5b-specific antibodies supershifted the IL-2-induced complex from control extracts, the patient extracts revealed significantly reduced reactivities to these antibodies (Fig. 3C, Lanes 4 and 5 versus Lanes 9, 10, 14, and 15). Thus, in contrast with healthy controls, IL-2 primarily induces the DNA binding of the Stat5 isoform in SzS patients. These results also
suggest that the difference in mobilities of the supershifted complexes could be attributed to the predominant presence of truncated rather than full-length Stat5 proteins in the IL-2-induced complexes from patients compared with control samples. These data suggest that the regulation of IL-2-induced Stat5-dependent activation of gene expression may be impaired in the SzS patients, because a significant proportion of the activated form of Stat5 potentially lacks the trans-activation domain.

Abrogation of IL-2-Induced Expression of Stat5 Target Genes in SzS Patients. To determine the functional consequences of the observed changes in Stat5 expression, we evaluated the expression of known Stat5-regulated genes by quantitative real-time reverse transcription-PCR, using mRNA isolated from preactivated PBMCs in two representative patients compared with three different control samples (Fig. 4). Three well-defined target genes of Stat5 were selected, Bcl-2, PIM1, and CISH, which are involved in survival, proliferation, and at negative regulation of cytokine receptor signaling (42–46).

In the three control samples expression of CISH and PIM-1 was induced within 30 min of IL-2 stimulation (Fig. 4, A and B). Although control 3 showed a reduced level of PIM-1 gene expression compared with the other two controls there was still a definite increase in expression after IL-2 stimulation (Fig. 4B). IL-2-induced Bcl-2 expression occurred at the later time point of 6 h after stimulation in each of the control samples (Fig. 4C). In contrast, in the two patients examined IL-2 stimulation resulted in minimal or no increase of each of the Stat5-dependent target genes (Fig. 4, A–C), suggesting that the predominant activation of Stat5, is unable to efficiently activate transcription of these target genes in these patients.

To confirm that these cells were not generally refractory to stimuli, we used the CD25 gene as a control, as this gene was shown previously to be regulated by constitutively activated Stat3 in SzS tumor cells (24). Consistent with these findings, we have also observed constitutive activation of Stat3 in PBMC of patients used in this study.2 As expected, the results of the real-time PCR for CD25 expression demonstrated IL-2-inducible activation of this gene in both control and patient samples, although at a slightly reduced level in patient samples compared with controls (Fig. 4D).

DISCUSSION

This study demonstrates that in T cells from healthy individuals there is differential expression of nuclear Stat5 isoforms depending on the activation status of the cells. This differential nuclear Stat5 isoform expression is dysregulated in SzS patients and consequently results in an abrogation of IL-2-induced Stat5-dependent target gene expression in peripheral blood malignant T cells from these patients.

The functional significance of the normal processing of Stat5 in mature PBMCs has, to date, not been clearly demonstrated. Although an earlier study observed the presence of a shorter M₇ 70,000–80,000 form of Stat5 proteins in fresh PBMCs, which changes to larger M₇ 96,000–94,000 forms of Stat5 proteins in preactivated PBMCs, the underlying molecular basis for this difference was not elucidated and was attributed to possible post-translational modification by phospho-
rylation events (10). The use of COOH-terminal Stat5a- and Stat5b-specific antibodies in this study establishes that the shorter form of Stat5 expressed in normal fresh PBMCs represents COOH-terminally truncated isoforms of Stat5, suggesting that this is the default status of Stat5 proteins in resting PBMCs. Thus, it seems likely that Stat5 expression is regulated by protein processing during the activation of peripheral T cells. Because the COOH terminus of STAT proteins contains the transactivation domain, this differential usage of functionally distinct isoforms of Stat5 may be an regulatory mechanism used by normal peripheral lymphocytes to prevent aberrant activation unless the appropriate activating signals are received (47–49).

The differential expression of Stat5 isoforms in PBMCs is reminiscent of a similar situation in murine myeloid cell differentiation and in terminally differentiated neutrophils (38–40, 50–52). These reports described COOH-terminally truncated isoforms of Stat5, which did not react with the COOH-terminal Stat5a and Stat5b antibodies, and failed to promote activation of Stat5-regulated genes. Extensive biochemical studies of myeloid cells have characterized an unidentified Mr ~25,000 putative serine protease whose activity is developmentally regulated during myelopoiesis (52, 53). However, these studies found no evidence for a similar protease in regulating T-cell development, and they were unable to detect the truncated Stat5 isoform in thymocytes or thymocyte-derived cell lines (53). A recent study has also demonstrated that calpain, a ubiquitously expressed cysteine protease, can cleave Stat5 in vivo and in vitro, within the COOH-terminal domain (54).

Although we have not precisely identified the site of the truncation of Stat5 proteins in fresh PBMCs, their size and lack of reactivity to COOH-terminal Stat5-antibodies suggests a similar truncation to that defined in myeloid cells and neutrophils. Given the similarity of the findings in control peripheral T cells from healthy individuals to that described previously for myeloid cells and neutrophils, it is highly likely that a similar serine protease may be responsible for the Stat5 processing observed in peripheral T cells. The cell fractionation analysis (Fig. 1B) revealed that the full-length Stat5 protein is indeed present in the cytoplasmic fraction of fresh PBMCs. However, only the Stat5 isoform appears in the nucleus of freshly isolated PBMCs, suggesting that full-length Stat5 proteins that translocate to the nucleus are also proteolytically processed in this compartment. Evidence for such a nuclear protease has been demonstrated previously in hematopoietic progenitors (55).

In light of these observations in normal PBMC, all five SzS patients displayed significant abnormalities in the regulated expression of the different isoforms of Stat5 in fresh versus activated PBMCs. Patient-derived nuclear extracts revealed significantly elevated levels of Stat5, in comparison with control samples in response to potent mitogenic stimulation. Although we compared Sezary cells, which are skewed to a T-helper 2 memory phenotype, to normal preactivated peripheral blood lymphocyte in this study, we have observed that normal T cells that have been differentiated in vitro to a T-helper 2 phenotype also express only full-length Stat5. Furthermore, unlike other hematopoietic cancerous cell lines and tumors, none of these patients revealed constitutive activation of Stat5. Together these results suggest that there is a selective pressure in the pathology of this disease to maintain a primarily inactive form of Stat5 in circulating malignant T cells from SzS patients as compared with other cancers. Although four of five patients showed some nuclear expression of full-length Stat5 proteins, these proteins were unable to activate IL-2-induced expression of Stat5-dependent genes as determined by quantitative PCR analysis (Fig. 4). This finding would suggest that Stat5, has a dominant-negative function, and competes for binding and transactivation with the full-length Stat5 protein. These findings are consistent with previous studies, which have shown that COOH-terminally truncated Stat5 proteins behave in a dominant-negative manner in in vitro transfection studies (50).

Importantly, quantitative PCR analysis (Fig. 4) also distinguishes “true” IL-2-inducible Stat5-target genes such as CISH, PIM-1, and Bcl-2 from a gene such as CD25 that can be regulated by both Stat5 and Stat3, and also activator proteins AP-1 (25, 28, 29, 46). Because both of these STAT proteins have overlapping and distinct functions in promoting cell cycle progression and regulation of apoptosis (23), the relative dysregulation of these proteins has important physiological implications for the survival and rate of expansion of malignant T cells in this disease.

The inability of SzS PBMCs to express a predominant level of functionally active full-length Stat5 proteins could provide one explanation for the indolent nature of this disease. Typically malignant cells in SzS patients proliferate poorly both in vivo and in vitro, which is probably a result of reduced cell-cycle progression. Stat5 proteins, which lack the transactivation domain, are unable to induce cell cycle-related genes in response to IL-2 treatment (56). Additionally, given the critical role of IL-2 in the maintenance of T-cell homeostasis by promoting deletion of mature, activated T cells, the predominant use of truncated Stat5 by malignant T cells in SzS may be one mechanism by which these cells escape apoptotic death (57–59). Because the SzS patients included in this study had aggressive disease and a high tumor burden as indicated by the high Sezary cell count, it is possible that these findings may be associated with advanced stage disease.

In conclusion, we provide the first example of dysregulated Stat5 isoform expression and function in malignant T cells from SzS patients. We suggest that the aberrant expression of the truncated isoform of Stat5 may be a key mechanism that promotes malignant T-cell transformation in SzS.

REFERENCES

DYSREGULATED STAT5 EXPRESSION IN SEZARY SYNDROME


Dysregulated Expression of COOH-Terminally Truncated Stat5 and Loss of IL2-Inducible Stat5-Dependent Gene Expression in Sezary Syndrome

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